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A KINETIC STUDY OF THE REACTION OF o-DIANISIDINE WITH HORSERADISH PEROXIDASE COMPOUND I AND COMPOUND II

by

(C) FARIDA MOHAMED SAAD EL-DIN EL-DARS

A THESIS

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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Date 22nd July 1985

TO: MY MOTHER, MY FATHER, AND MY BROTHERS.

ABSTRACT

The kinetics of the oxidation of o-dianisidine by compound I and compound II of horseradish peroxidase were investigated as a function of pH by the stopped-flow technique. The enzyme intermediates involved in the reactions were studied by rapid scan spectrometry. The second order rate constant for the reaction of o-dianisidine with compound I was independent of the pH between pH 7.91 and pH 10.60 ($\simeq 10^8$ $M^{-1}s^{-1}$ at 10.0°C). At pH lower than 7.91, the rate increased beyond the stopped-flow time scale. A plot of the pseudo-first order rate constant for the reaction of compound II with o-dianisidine versus the o-dianisidine concentration was not linear. Therefore, it was concluded that the reaction occurred in two steps. The first step involves the fast binding of the substrate to the enzyme forming an enzyme-substrate complex. The complex undergoes a conformational change or an intramolecular rearrangement to yield the free product and native enzyme. At high concentrations, the second step becomes rate limiting. The existence of the complex was confirmed by spectrophotometric measurements. The rate of the reaction of o-dianisidine with compound II decreased with increasing the pH indicating the influence of an enzyme ionizing group with $pK_a \simeq 8.6$.

PREFACE

Horseradish peroxidase has been extensively studied over the last forty years. The intermediates (compound I and compound II) of the enzymatic cycle have been identified and studied in great detail (for a review see reference 1).

o-Dianisidine (3,3'-dimethoxybenzidine) has been frequently used as a probe for the detection of peroxidase activity and peroxide generating systems. The chemical intermediates and products of the peroxidation of o-dianisidine by horseradish peroxidase have been identified. The final product was found to be biazobiphenyl (2,3).

The present study concentrates on the reactions of compound I and compound II with o-dianisidine. The stopped-flow technique has been used to study the kinetics of both reactions. The enzyme intermediates were identified by rapid scan and conventional spectrophotometry. In the introductory chapter, some properties of peroxidases in general and horseradish peroxidase in particular are presented. A summary of the existing literature on the peroxidase catalyzed oxidation of o-dianisidine is also included. Chapter II contains the experimental procedures. In chapter III, the results of the kinetic studies are presented and analyzed. In the final chapter, the results are discussed and compared to data from the literature.

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I. INTRODUCTION

A. PEROXIDASES

Peroxidases (*E.C.1.11.1.7,donor: hydrogen peroxide oxidoreductase*) are a class of enzymes that were discovered in 1855 by Schoenbein (4). Then, it was reported that certain 'substances' occurring in plants and animals were found to catalyze the oxidation of certain organic compounds in the presence of dilute solutions of hydrogen peroxide. The name 'peroxidase' was first given to these 'substances' by Linossier in 1898 who was able to isolate an oxidase-free preparation of peroxidase from pus (5). Peroxidases are widely distributed in nearly all plant cells especially in higher plants (6,7). High concentrations of the enzyme occur in the sap of the fig tree and in the root of the horseradish plant. Peroxidases are also present in many animal tissues and fluids such as in the adrenals, in the liver, in the kidney, and in the thyroid glands (thyroid peroxidase), also in the blood (from leucocytes one can obtain myeloperoxidase), in milk (lactoperoxidase), and in the saliva (8,9a). Other peroxidases are present in fungi (e.g. Pencillium chrysogeum) and in bacteria (e.g. Pseudomonas aeruginosa) (9a).

Peroxidases of mammalian origin have diverse physiological functions, for example, myeloperoxidase and

lactoperoxidase have antibacterial functions (9b) while thyroid peroxidase catalyzes the iodination of the protein thyroglobulin to yield the formation of the thyroid gland hormones (9c,10). The functions of the plant peroxidases are not clearly understood. This is mainly due to the inability of scientists to identify a specific natural substrate for them. In laboratory conditions, plant peroxidases catalyze the oxidation of a variety of organic compounds and inorganic ions such as phenols, aromatic amines, iodide, sulphite, nitrite, and ferrocyanide in the presence of hydrogen peroxide, alkyl peroxides, and peracids(1).

The prosthetic group of most plant peroxidases is protohaematin IX (also known as ferriprotoporphyrin IX)(Figure I.1.). The protein moiety of the enzyme is called the apo-enzyme. It is generally accepted that the protein moiety and the prosthetic group are essential not only for the catalytic action but also for the specificity of the enzyme (11,12).

Over the last half of a century, horseradish peroxidase has become one of the more extensively studied peroxidases. This is due to its ready availability and its high purity and stability upon isolation. Moreover, horseradish peroxidase has the ability to mimic the catalytic action of some mammalian enzymes of physiological importance; e.g. thyroid peroxidase, that are not easily isolated and can not be studied due to their high instability (10).

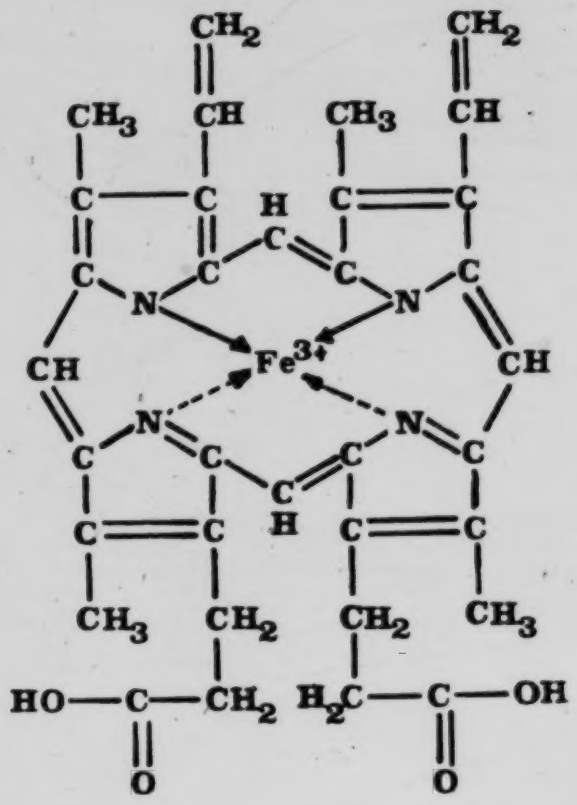

Figure I.1. Structure of protohaematin IX.

B. HORSERADISH PEROXIDASE

1. Structure of Horseradish Peroxidase

Horseradish peroxidase is known to have seven major (13) and fourteen minor isoenzymes (14). The seven major isoenzymes are classified into: acidic (A1,A2,A3), neutral (B and C), and basic (D and E) components (13). The different isoenzymes could be easily separated by analytical techniques such as salt precipitations, paper electrophoresis, or chromatography due to the differences in their isoelectric points (8). Isoenzyme C (in the older literature sometimes referred to as III b) has an isoelectric point close to 9 and is relatively high in abundance compared to other isoenzymes in horseradish roots (15).

Horseradish peroxidase is a monomeric glycohemoprotein of molecular weight 44,000 (15). It has 43% α-helix structure(16), and a carbohydrate content of 18% by weight (17). Amino acid sequencing (17) indicates the enzyme consists of a single polypeptide chain comprised of 308 amino acid residues. The enzyme contains one protohaematin IX and eight neutral carbohydrate side chains attached through asparagine residues. Also present are four disulphide bridges and three histidyl residues (His 40,His 42,His 170). One of the histidines (namely His 170) was

found to occupy the fifth coordination position of the ferric ion (18) while the other two residues (His 40,His 42) are present in the distal side of the heme (19). His 42 is considered to work as the distal histidine group of catalytic importance (19). The presence or absence of a water molecule in the sixth coordination position of the heme has been debated. A recent Raman spectroscopy study of the enzyme has lead to the proposal that the sixth coordination position of the heme iron of native ferric horseradish peroxidase is vacant, but upon binding of a particular substrate (in the study the substrate was benzhydroxamic acid) a water molecule is accommodated in the axial coordination position of the heme (20). This was concluded from the comparison of the Raman spectra of the native ferric horseradish peroxidase which was that of a penta-coordinated ferric high spin heme and that for the horseradish peroxidase-benzhydroxamic acid complex which was typical of a hexa-coordinated structure.

The presence of two calcium ions per enzyme molecule was detected (21). The binding of one calcium ion to the enzyme was found to be essential in maintaining the protein structure in the heme enviroment. It appears to stabilize the higher oxidation state of the heme in compound I of horseradish peroxidase (22).

2. The Enzymatic cycle of Horseradish Peroxidase

There are two enzymatic intermediates in the catalytic cycle of horseradish peroxidase (compound I and compound II). The formation of compound I is achieved by the reaction of hydrogen peroxide with the native ferric enzyme. During this reaction, the enzyme acquires an oxygen atom from hydrogen peroxide. Therefore, compound I has a formal oxidation state of +5. The one-electron reduction of compound I by the reaction with a substrate yields compound II. Therefore, compound II has an intermediate oxidation state of +4 between compound I and the native ferric enzyme. The three species (native enzyme, compound I, and compound II) have very distinguishable Soret peaks, and the study of the formation and decay of each intermediate is rendered feasible due to the presence of distinct isosbestic points (Figure I.2.).

Studies have indicated that the enzymatic cycle for horseradish peroxidase is dependent upon the nature of the reducing substrate. For the horseradish peroxidase-catalyzed oxidation of ferrocyanide (24,25), *p*-aminobenzoic acid (26), and nitrite (27), the following scheme was found to be representative of the series of reactions occurring within the cycle:

$$HRP + H_2O_2 \longrightarrow HRP\text{-}I + H_2O \qquad (1)$$

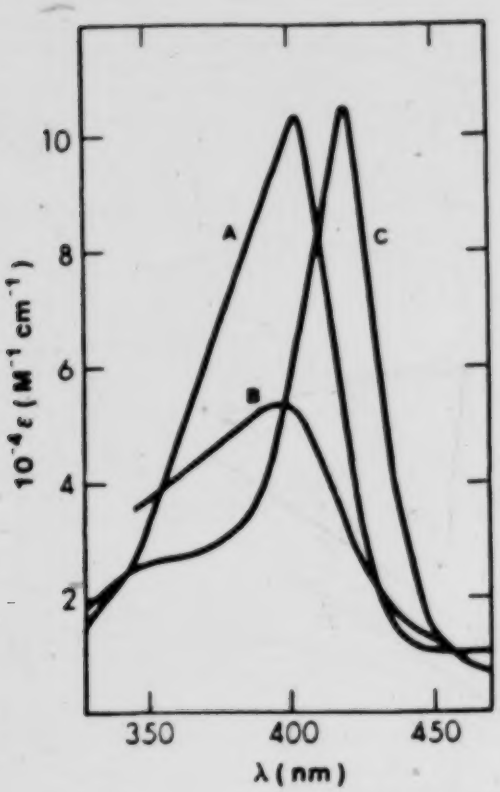

Figure I.2. Molar Absorptivity in the Soret region of Horseradish peroxidase (23):
(A) native enzyme
(B) compound I, and
(C) compound II .

$$HRP\text{-}I + S \longrightarrow HRP\text{-}II + S' \qquad (2)$$

$$HRP\text{-}II + S \longrightarrow HRP + S' \qquad (3)$$

where HRP, HRP-I, HRP-II, S and S' are the native ferric enzyme, compound I, compound II, the reducing substrate and the product, respectively.

For the oxidation of *p*-cresol, (28,29), the following scheme applies :

$$HRP + H_2O_2 \longrightarrow HRP\text{-}I + H_2O \qquad (1)$$

$$HRP\text{-}I + HS \longrightarrow HRP\text{-}II + S' \qquad (4)$$

$$HRP\text{-}II + HS \longrightarrow \{HRP\text{-}II\text{-}HS\} \longrightarrow HRP + S' \qquad (5)$$

where HS, HRP-II-HS, and S' represent the substrate, the enzyme-substrate complex and the substrate radical, respectively.

In some other reactions of horseradish peroxidase, compound II was not detected within the enzymatic cycle even though it itself was found to react with the substrates. This was evident in the oxidation of iodide (30) and bisulphite (27). The suggested reaction scheme is:

$$HRP + H_2O_2 \longrightarrow HRP\text{-}I + H_2O \qquad (1)$$

$$HRP\text{-}I + S \longrightarrow HRP + S'' \qquad (6)$$

where S" is the two-equivalent oxidized form of the substrate.

Apart from the three main species of horseradish peroxidase that are actively involved in the enzymatic cycles, another redox form has been studied (compound III). Compound III can be formed by the addition of excess hydrogen peroxide to the native ferric enzyme (31) or by the binding of oxygen to the ferrous enzyme (7). This species is catalytically inactive and the peroxidase activity is inhibited due to its formation (7,31).

3. Compound I and Compound II: Structure and Reactivity

Both compound I and compound II have the same ferryl (Fe(IV)) structure (32). However, in compound I, there are two oxidizing equivalents: one equivalent stored as a low-spin ferryl ion, and the second equivalent stored as a porphyrin centered π-cation radical (33). In compound II, there is only one oxidizing equivalent present. The reactions of compound II are generally slower than those of compound I (7). The reactions of compound I appear to be influenced by an enzyme group of a $pK_a \simeq 5.1$, whereas the reactions of compound II appear to be influenced by another enzyme group of $pK_a \simeq 8.6$ (1). The protonation of the enzyme group of $pK_a \simeq 8.6$ in compound II has a noticable effect upon the oxidation rates of most substrates studied such as

p-cresol, ferrocyanide and *p*-aminobenzoic acid. However, in other reactions of compound II with substrates such as iodide and bisulphite, another enzyme group having a pK_a lower than 3 greatly affected the observed rates of oxidation. The importance of the enzymatic group of $pK_a \simeq 8.6$ will be discussed further in chapter IV.

C. PEROXIDASE CATALYZED OXIDATION OF o-DIANISIDINE

Horseradish peroxidase has been widely exploited for the detection of hydrogen peroxide and hydrogen peroxide generating systems in analytical biochemistry (34,35). The oxidation of o-dianisidine by horseradish peroxidase was first studied as a basis for a convenient and specific assay for the determination of glucose (2). The basis of the assay is the oxidation of glucose by glucose oxidase to yield gluconolactone (D-gluconic acid) and hydrogen peroxide. In the presence of horseradish peroxidase, the hydrogen peroxide produced is utilized in the oxidation of o-dianisidine to a coloured product. This method was further extended and used for the determination of carbohydrates especially sugar in human blood and urine (36) and for peroxidase determination in various mammalian tissues(37).

The horseradish peroxidase /o-dianisidine oxidation system was applied in histochemistry and neuroanatomy. Horseradish peroxidase neurohistochemistry is one of the most frequently used methods of tracing neuronal connectivity within the central nervous system (38-44). This is due to the ready uptake of horseradish peroxidase by nerve cells and its subsequent transportation through the central nervous system from the original place of injection under the skin to the brain. The detection of the path undertaken by HRP can be easily visualized using chromogens

(aromatic amines) which can polymerize and show a more intense colour when oxidized (36-44). One of the most efficient chromogens used for this method is o-dianisidine. The optimum pH for the peroxidation of o-dianisidine was found to be within the pH range of 3.4 - 6.4 (40). The o-dianisidine /horseradish peroxidase system was employed also in the determination of the peroxidatic activity of the enzyme itself (45).

Despite the extensive application of the o-dianisidine oxidation by horseradish peroxidase in various analytical fields, initially little attention was paid to the study of the reaction itself. A number of studies were published concerning the fate of o-dianisidine in the peroxidation cycle (2,3,46). In the earliest of these studies (46), the enhancement of the rate of the peroxidation of o-dianisidine using ammonium salts was investigated. In the absence of NH_4^+, the rate of peroxidation decreased above pH 5.8, and in the presence of NH_4^+, the rate only decreased when the pH exceeded 8.8. In the present study, all experiments were performed in the absence of ammonium salts. Therefore, the process responsible for the rate enhancement by nitrogenous ligands will not be discussed further here. The rate limiting step for the overall reaction was believed to be the reaction of compound II of horseradish peroxidase with o-dianisidine. The overall reaction scheme was given as follows:

$$HRP + H_2O_2 \longrightarrow HRP\text{-}I \qquad (7)$$

$$HRP\text{-}I + DH_2 \longrightarrow HRP\text{-}II + DH \qquad (8)$$

$$HRP\text{-}II + DH_2 \longrightarrow HRP + DH \qquad (9)$$

where DH_2 and DH are the substrate and the substrate radical, respectively.

The more recent studies (2,3) focused on the mechanism of the oxidation of o-dianisidine and the identification of the final products. It was concluded that the oxidation of o-dianisidine occurs in a rapid two-electron process(2). At low concentrations of the substrate, the first free detectable product was identified as the two-equivalent oxidized form of o-dianisidine which is dianisidine quinonediimine (Figure I.3.). With the concentration of the substrate in excess of that of the enzyme, the first detectable product was an intermolecular complex consisting of the quinonediimine and o-dianisidine. The final product was identified as biazobiphenyl(structure Figure I.4.). The interaction of the o-dianisidine molecule with the native enzyme was also studied (3). Evidence for the formation of an enzyme-substrate complex was reported and the importance of this finding will be elaborated upon in chapter III and chapter IV.

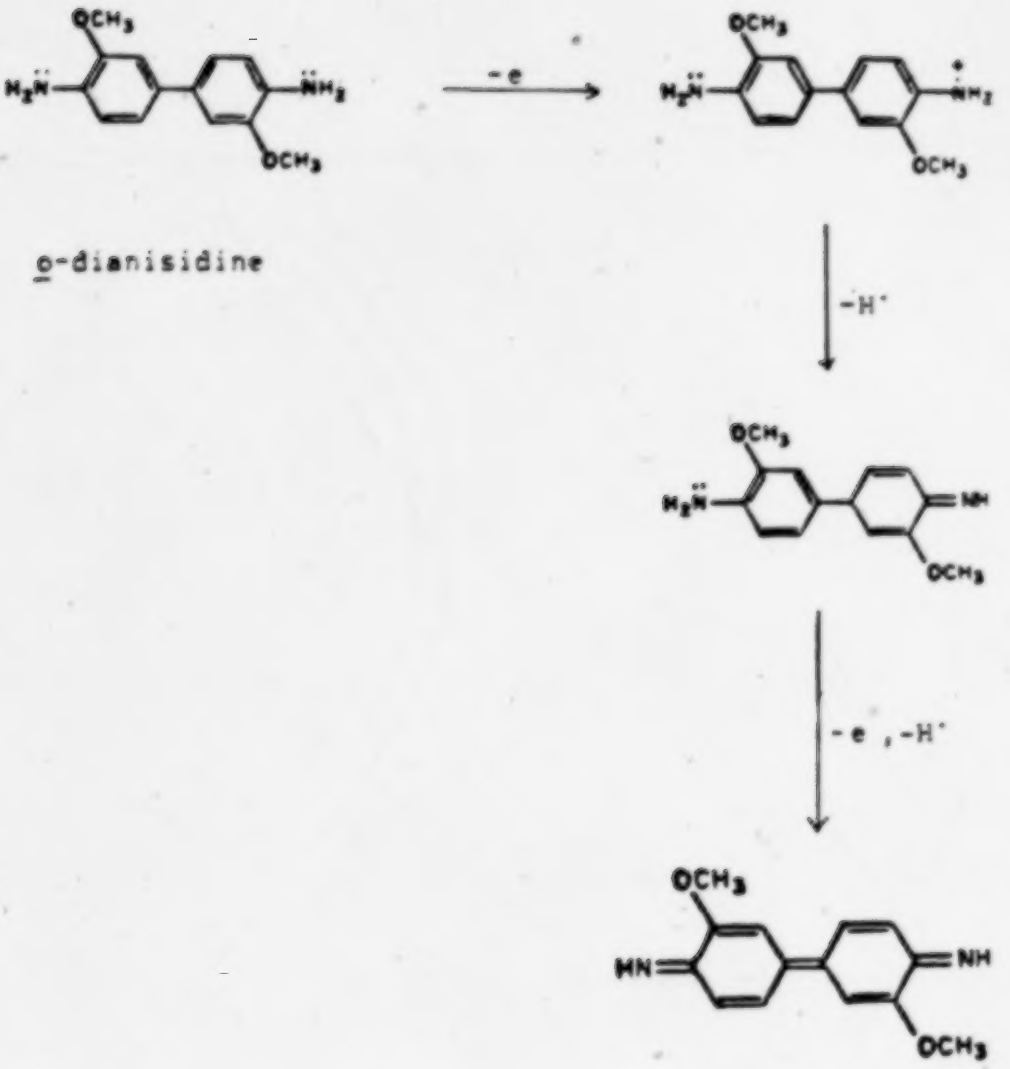

Figure I.3. Oxidation of o-Dianisidine to Dianisidine Quinonediimine.

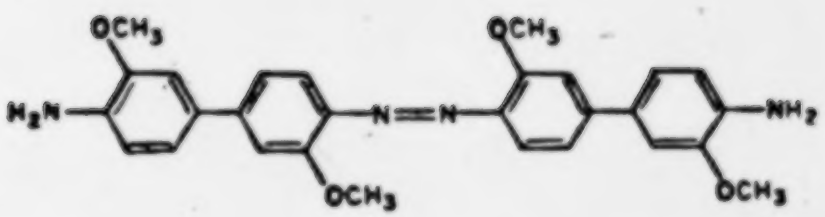

Figure I.4. Structure of Biazobiphenyl.

II. EXPERIMENTAL

Horseradish peroxidase (*HRP*) (*E.C.1.11.1.7,donor: hydrogen peroxide oxidoreductase*) was obtained from *Boehringer Mannheim Corp.(Montreal)* as an ammonium sulphate precipitate with a purity number (*ratio of absorbances at 403 nm and 280 nm*) of 3.00. Stock solutions of the enzyme were prepared by dialyzing the precipitate extensively at 4°C against purified distilled water obtained from a *Milli-Q Reagent-Grade Water System*. The concentration of the enzyme was determined spectrophotometrically at 403 nm using a molar absorptivity of 1.02×10^5 $M^{-1}cm^{-1}$ (47). o-Dianisidine (3,3'-dimethoxybenzidine) dihydrochloride (*o-DH*$_2$) was obtained as purified crystals from *Sigma (St. Louis)*. Because of its low solubility in water, a stock solution of o-dianisidine was prepared by dissolving 0.3188 g in 200 ml 95% ethanol to yield a concentration of 5 mM. All solutions made of o-dianisidine (stock and further dilutions) were protected from light and stored at 5°C to prevent oxidation (37).

Hydrogen peroxide (30%) was obtained from *Fisher (Fair Lawn, New Jersey)*;and the concentration of H_2O_2 stock solution was checked spectrophotometrically by *HRP* catalysis of the conversion of I^- to I_3^- (48). Potassium ferrocyanide (reagent grade $K_4Fe(CN)_6.3H_2O$) was obtained from *Baker Chemical Co. (Phillipsburg, N.J.)*. Potassium ferrocyanide

solutions of the required concentration were prepared by dissolving a carefully weighed amount in reagent grade water. All other chemicals used as buffer components were of reagent grade and were used without further purification.

Spectrophotometric measurements were performed on a *Cary 219* spectrophotometer with the cell compartments thermostated at 25.0°C. All stopped-flow and rapid scan measurements were performed on a *Union Giken Rapid Reaction Analyzer Model RA-601* equipped with a 1-cm cell thermostated at 10.0±0.2°C. Measurements were made in buffered solutions of a total ionic strength of 0.11M; with the buffer contributing 0.01M. The final ionic strength was adjusted to 0.11M by adding potassium sulphate (pH 4.47 - pH 7.00) or potassium nitrate (pH 7.00 - pH 10.60) as inert salts. The concentration of *HRP* in the solutions was 1.0 - 2.0μM.

Compound I of horseradish peroxidase (*HRP-I*) was formed prior to use by adding an equimolar amount of hydrogen peroxide to 3.0 ml of the enzyme solution. The reaction of o-dianisidine and compound I was studied under second-order conditions using equimolar amounts of o-dianisidine and HRP. The reaction was monitored at 414 nm, the isosbestic point between the native enzyme and compound II.

Compound II of horseradish peroxidase (*HRP-II*) was formed just prior to use by adding an equimolar amount of hydrogen peroxide to 3.0 ml of the enzyme solution containing an equimolar amount of potassium ferrocyanide. For experiments within the pH range of 7.20-10.60, the

enzyme solutions were buffered to an ionic strength of 0.11M, and for experiments within the pH range of 4.47-7.00, a pH jump procedure was followed (49). The solutions after each experiment were collected and their final pH values were determined with a *Fisher digital pH meter (model 420)* equipped with a *Fisher MicroProbe combination* electrode. To ensure pseudo-first order conditions for the stopped-flow experiments, o-dianisidine concentration was at least 5 fold greater than that of compound II. The reaction between compound II of horseradish peroxidase and o-dianisidine was followed by monitoring the rate of disappearance of compound II at 420 nm, the wavelength of its maximum absorbance. Between 7 and 14 traces of change in absorbance versus time were recorded for each reaction.

III. RESULTS

An overall visualization of the enzymatic cycle could be obtained from the rapid scan spectra. The spectra in Figures III.1. and III.2. revealed that the reaction between compound I of horseradish peroxidase and o-dianisidine is extremely fast (14-15 ms upon completion) in the presence of a 5 fold excess of o-dianisidine. The reaction of compound II of horseradish peroxidase and an approximate five fold excess of substrate (Figures III.1. and III.3.) is slower and dependent upon the experimental pH (≃120 ms at pH 10.60 and ≃54 ms at pH 9.80). Figure III.2. illustrates that the addition of half the equimolar amount of the substrate to compound I yields the formation of compound II (100%, maximum absorbance peak at 420 nm) in a period of 27 ms at pH 10.60.

From Figures III.1. and III.3., the isosbestic point between native HRP and HRP-II was obtained at 414 nm. Under second-order conditions with equal concentrations of HRP and o-dianisidine, the kinetics of the reaction were analyzed according to the following differential rate expression:

$$-\frac{d\,[\text{HRP-I}]}{dt} = k'[\text{HRP-I}][\text{o-dianisidine}] \qquad (10)$$

The second-order rate constant k' was calculated from the initial slope of curves such as in Figure III.4. The mean

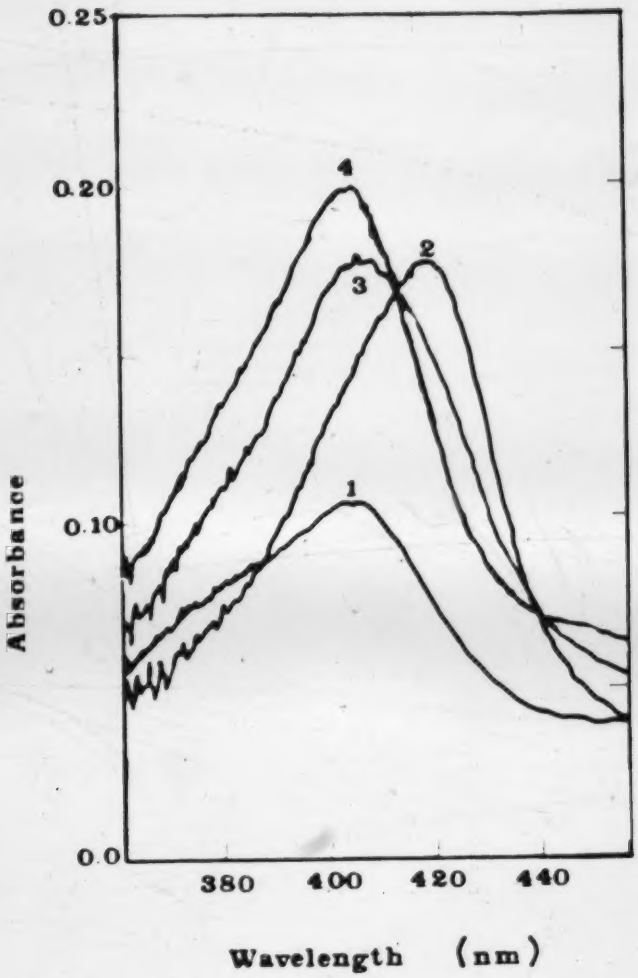

Figure III.1. Rapid scan measurements of the reaction of o-dianisidine with HRP-I at pH 10.60, with [HRP]= 1.73μM, and [o-dianisidine]= 8μM. Spectrum #1: HRP-I at t=0 ms, spectrum #2: HRP-II at t= 14 ms, spectrum #3 : the reaction at t= 38 ms , and spectrum #4: 158 ms after.

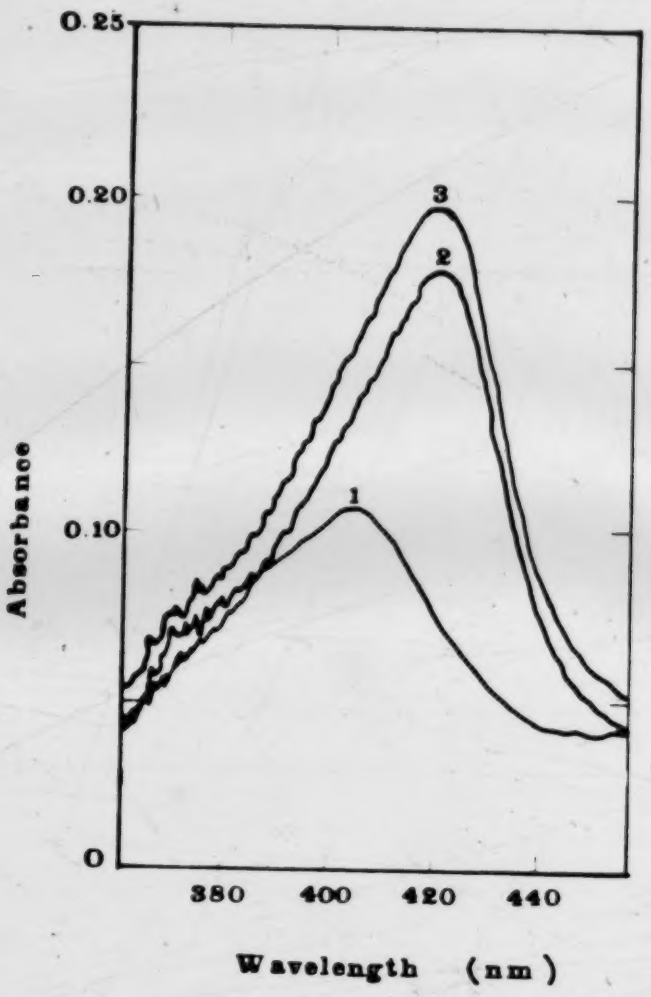

Figure III.2. Rapid scan measurements of the reaction of o-dianisidine with HRP-I at pH 10.60 ,with [HRP]= 1.73μM, and [o-dianisidine] = 1.0μM . Spectum #1 : HRP-I at t= 0 ms, spectrum #2 : the reaction after t = 3 ms , and spectrum #3 : the reaction after t= 27 ms .

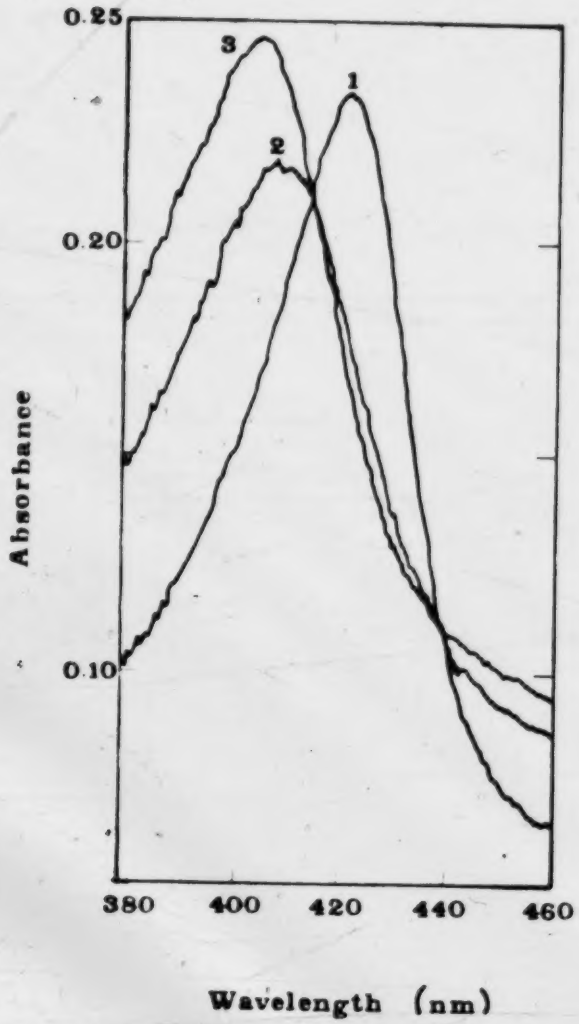

Figure III.3. Rapid scan measurements of the reaction of o-dianisidine with HRP-II at pH 9.80 with [HRP]= 2.05μM and [o-dianisidine]= 10μM . Spectrum #1 : HRP-II at t =0 ms, spectrum #2 : the reaction after t= 6 ms , and spectrum #3: t= 54ms after.

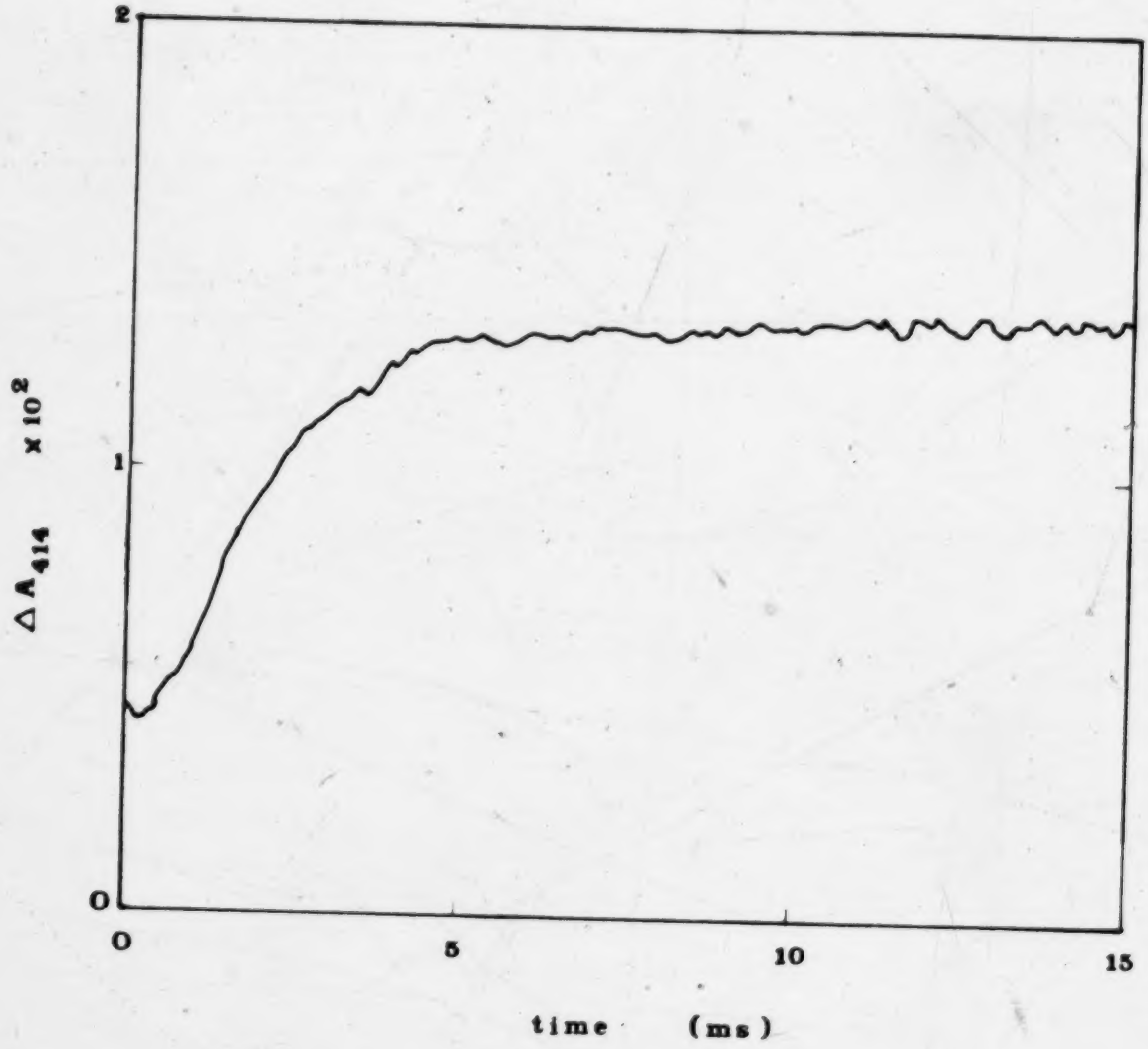

Figure III.4. A typical second-order trace of the reaction of HRP-I with o-dianisidine at 414 nm.

values of k' obtained are presented in Table III.1., and a plot of log k' versus pH is shown in Figure III.5.

Figure III.6. illustrates a typical pseudo-first order curve of ΔA (change in absorbance) versus time (msec) for the reaction of compound II with o-dianisidine. Under pseudo-first order conditions with the concentration of the substrate in excess that of the enzyme, the kinetics of the reaction were analyzed according to the differential rate expression (50,51):

$$- \frac{d\,[HRP\text{-}II]}{dt} = k(obs)[HRP\text{-}II] \qquad (11)$$

such that,

$$k(obs) = k(app)[o\text{-dianisidine}] \qquad (12)$$

where k(obs) is the observed rate constant, and k(app) is the apparent second-order rate constant. The observed rate constant for each experiment was calculated by a non-linear least-squares computer program using Gauss' method (52). Plots of k(obs) versus [o-dianisidine] were constructed at each experimental pH value and the results are shown in Figures III.7. and III.8.

Table III.1.

Values of k' for the reaction of HRP-I and o-dianisidine.

pH	$k'(M^{-1}s^{-1})$	log k'
7.91	$(1.4\pm0.3)\times10^{8}$	8.15
9.12	$(1.3\pm0.3)\times10^{8}$	8.10
9.73	$(1.2\pm0.1)\times10^{8}$	8.09
10.33	$(1.1\pm0.4)\times10^{8}$	8.05
10.60	$(1.0\pm0.1)\times10^{8}$	8.02

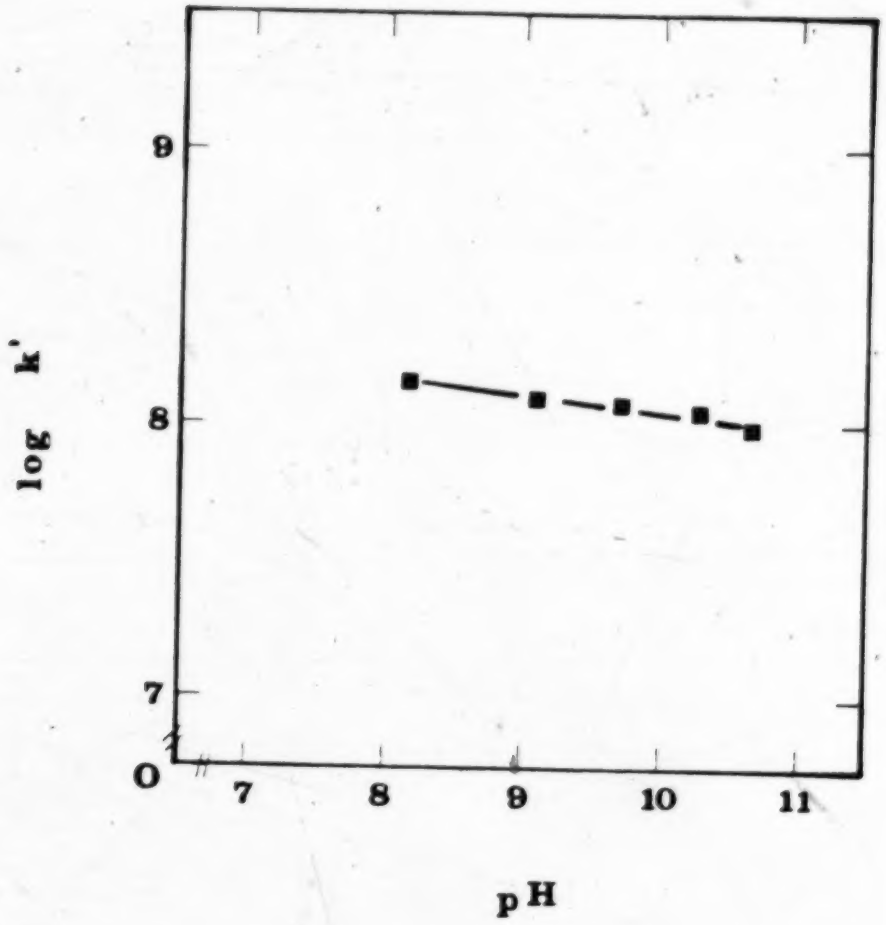

Figure III.5. Log k' versus pH for the reaction of HRP-I with o-dianisidine at 10.0±0.2°C.

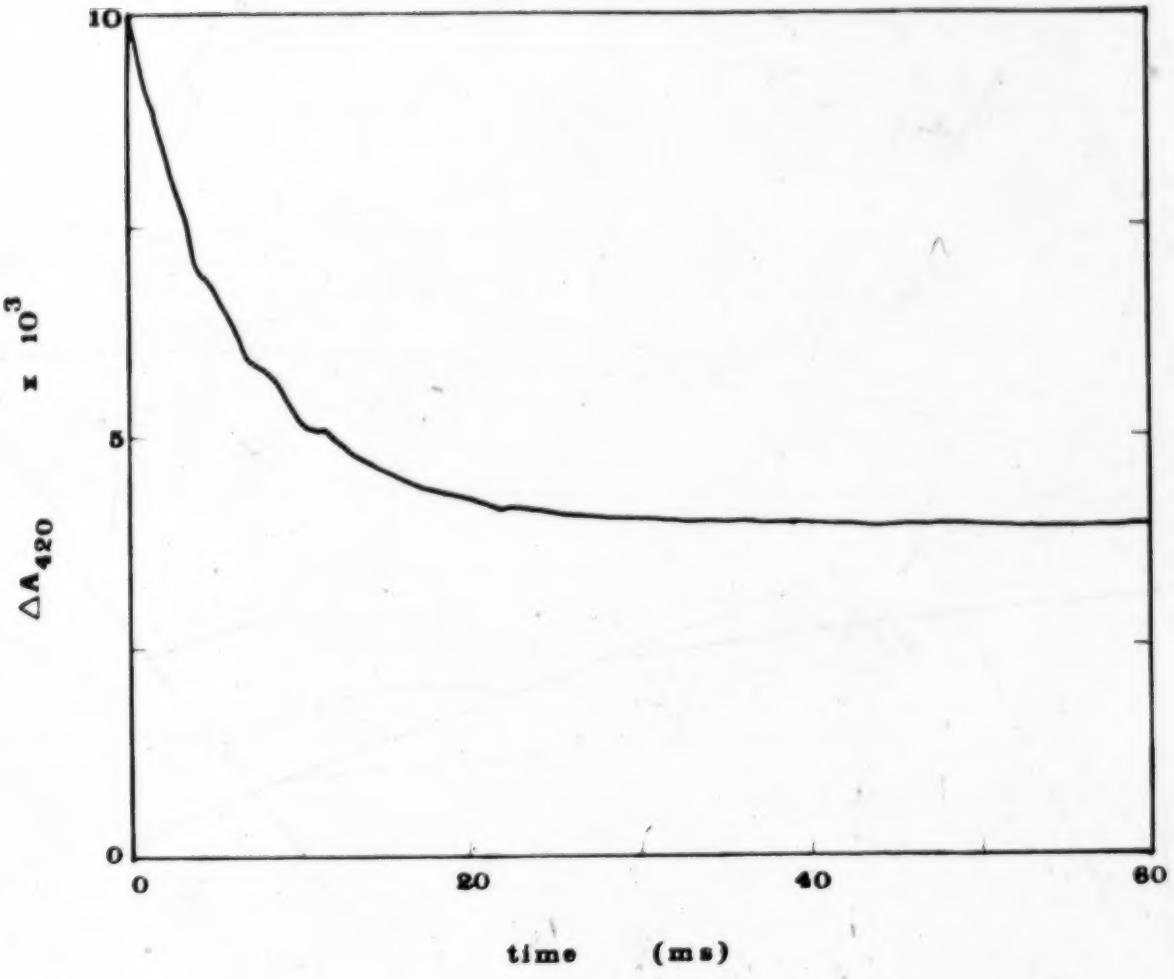

Figure III.6. A typical pseudo-first order trace of the reaction of HRP-II and o-dianisidine, at 420 nm and pH 8.20 with [HRP]= 1.2μM and [o-dianisidine]= 8μM.

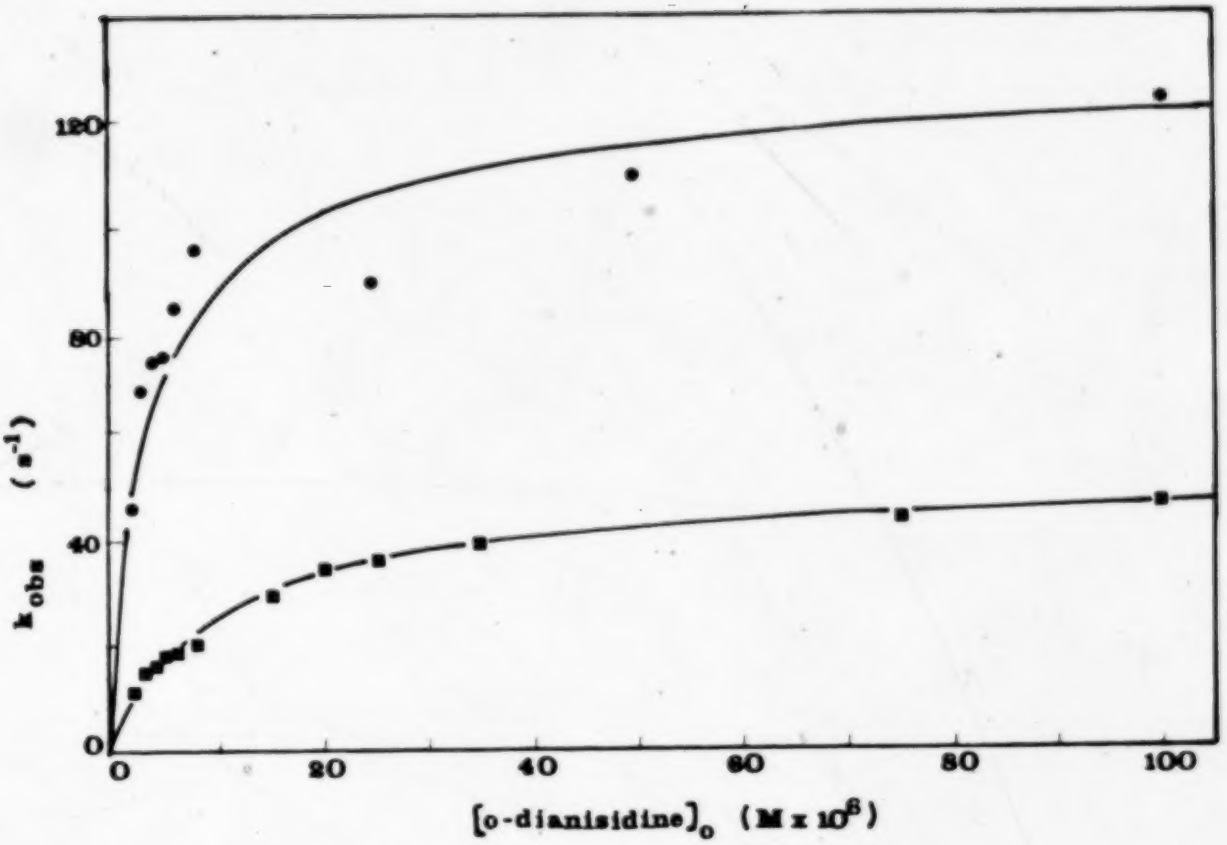

Figure III.7. Plots of k(obs) versus [o-dianisidine] for the reaction of o-dianisidine with HRP-II:
(●) at pH 9.00, and
(■) at pH 10.60.
[HRP] = 0.6μM after mixing.

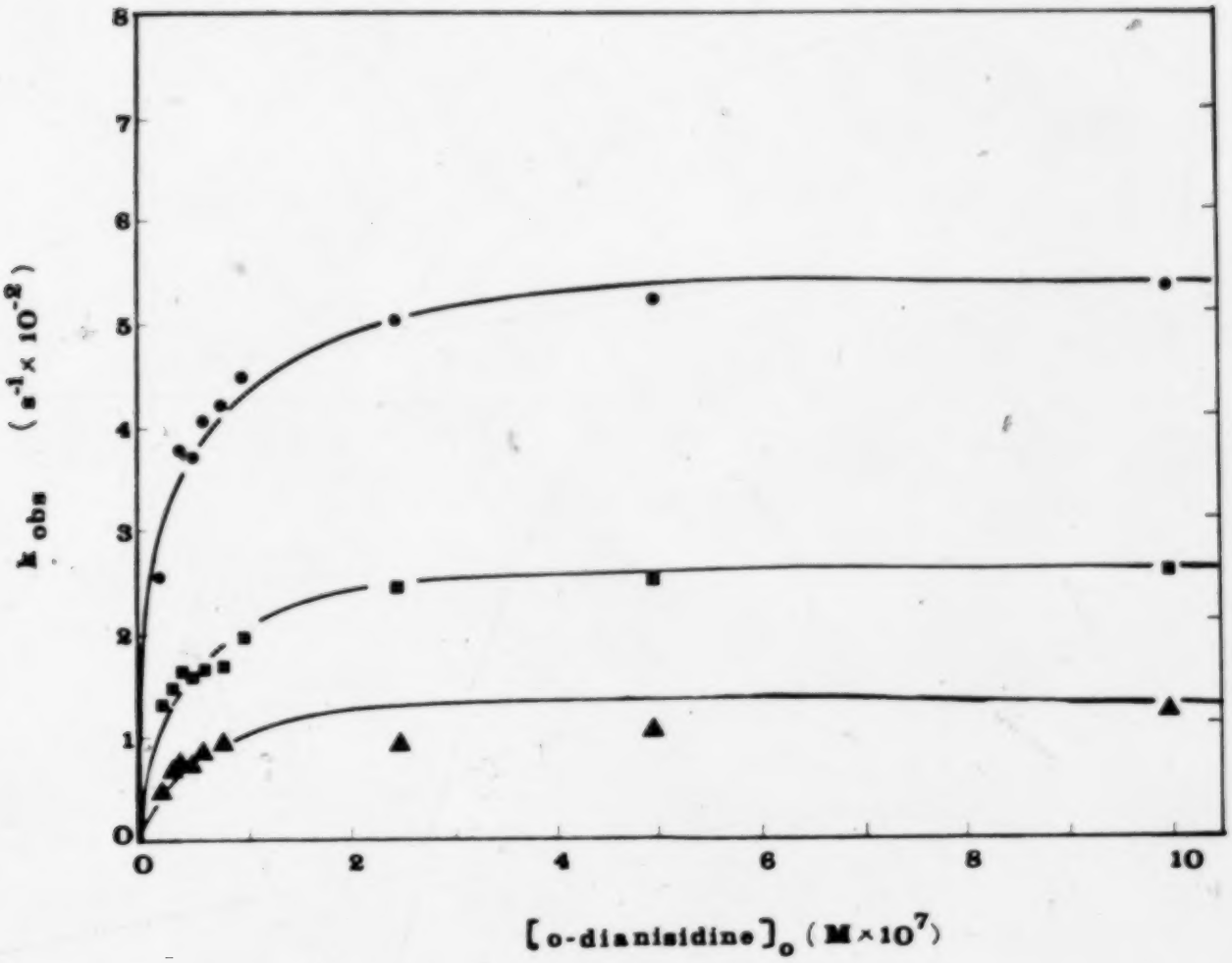

Figure III.8. Plots of k(obs) versus [o-dianisidine] for the reaction of o-dianisidine with HRP-II:
(●) at pH 7.60,
(■) at pH 8.20, and
(▲) at pH 9.00.
[HRP] = 0.5 -0.6 μM after mixing.

The linear relationship between k(obs) and [o-dianisidine] predicted by equation 12 was observed only at low concentrations. At higher concentrations, a non-linear dependence was observed. A similar behavior was reported in the oxidation of ferrocytochrome C (53) and in the oxidation of *p*-cresol (28) by compound II and was attributed to the formation of an enzyme-substrate complex.

The binding of the substrate to the enzyme could be detected spectrophotometrically as shown in Figures III.9. and III.10. The initial spectra of the native enzyme were recorded prior to the formation of the enzymatic intermediates: HRP-I (Figure III.9.) and HRP-II (Figure III.10.). The spectra were then recorded immediately after the addition of an equimolar amount of o-dianisidine to the enzymatic intermediates. An increase in the absorbance above the native enzyme peak (at 403 nm) was observed as well as the appearance of a broad shoulder at 450 -550 nm. These results are in agreement with the results previously reported (3). The increase in the absorbance peak was attributed to the binding of a partially oxidized substrate to the active site of the enzyme (3).

From the analysis of the results and data obtained from the stopped-flow experiments, the oxidation of o-dianisidine by compound II can be represented as a two step reaction mechanism (3,28,54-56). The first step involves a fast equilibrium binding of o-dianisidine to compound II. The second step involves an intramolecular rearrangement or a

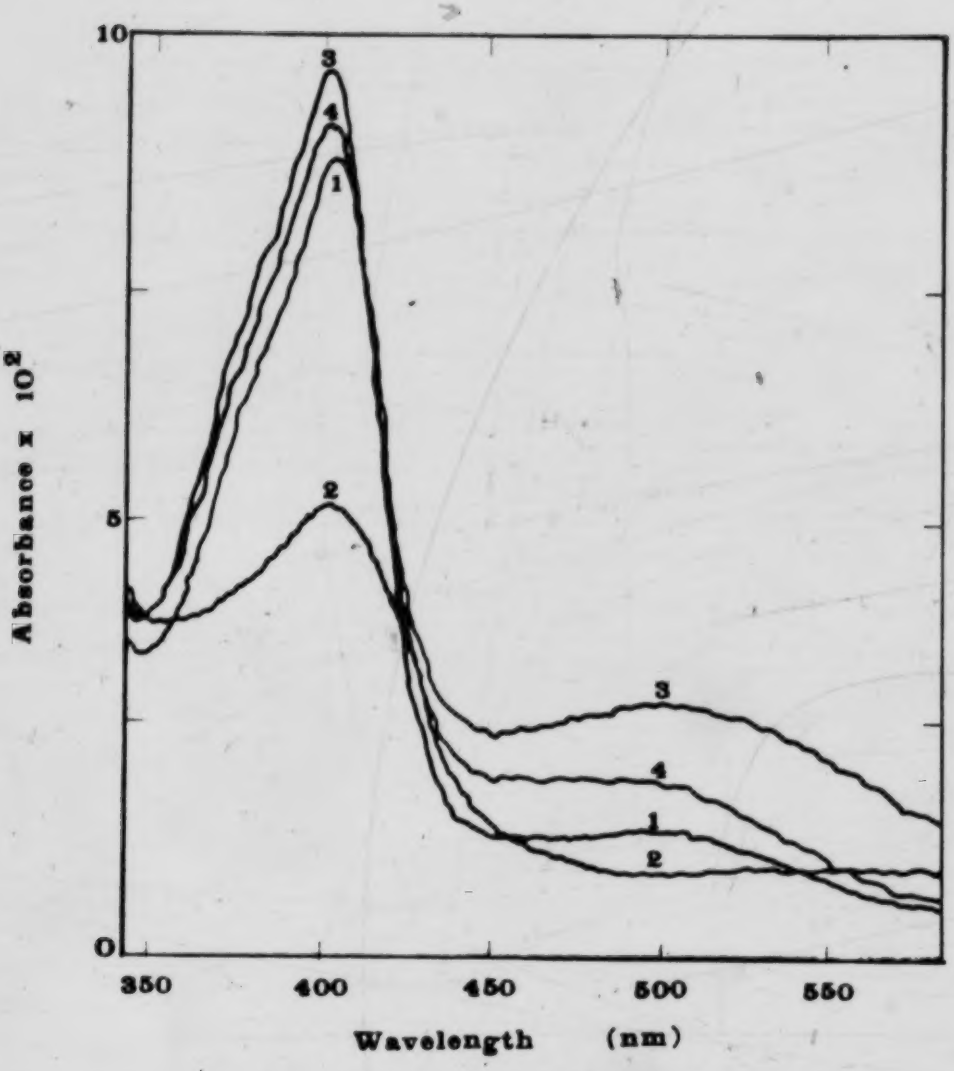

Figure III.9. Spectrophotometric measurements of the reaction of o-dianisidine with HRP-I at pH 7.60 ,and [HRP]= 1.0μM. Specrum #1 : native enzyme, spectrum #2 : HRP-I , spectrum #3 : the first spectrum obtained after the immediate addition of an equimolar amount of o-dianisidine to HRP-I (scan rate= 5nm/s), and spectrum #4 : 170s after mixing of HRP-I and o-dianisidine.

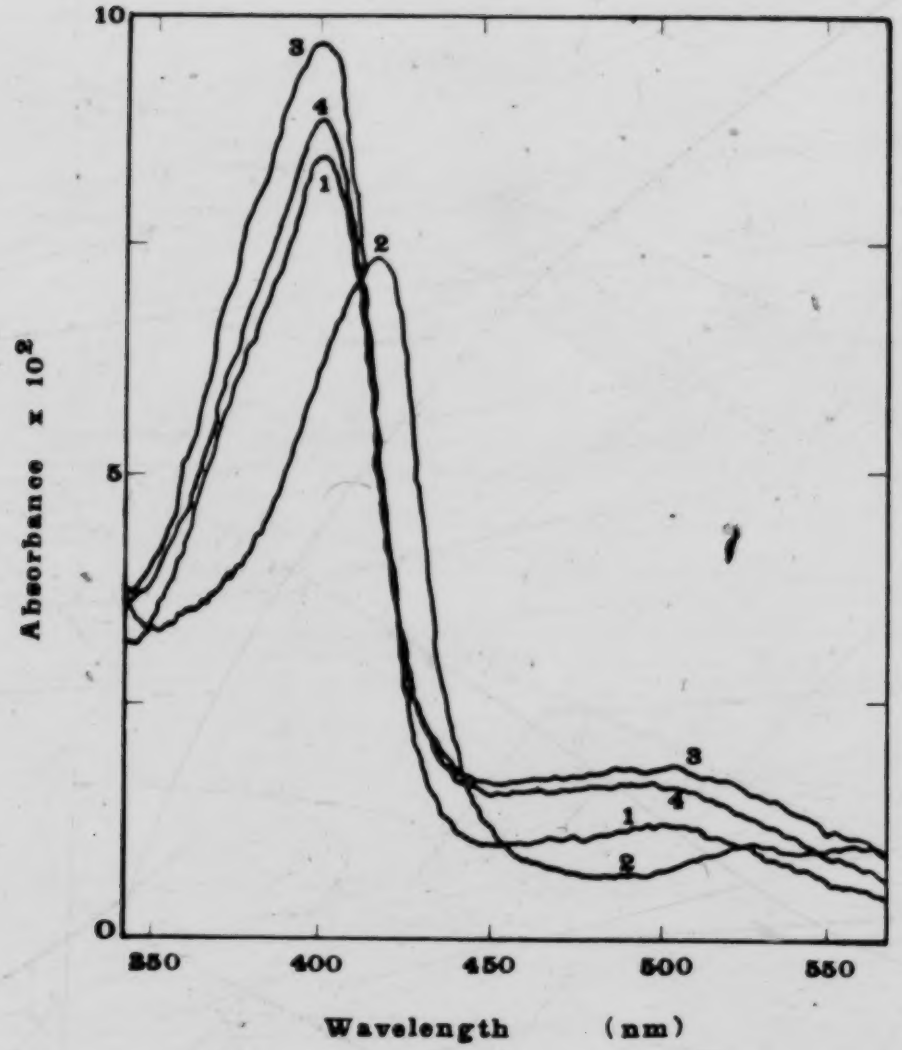

Figure III.10. Spectrophotometric measurements of the reaction of o-dianisidine with HRP-II of at pH 7.60 and [HRP]= 1.0μM . Spectrum #1: native enzyme , spectrum #2 : HRP-II , spectrum #3 :the first spectrum obtained after the immediate addition of an equimolar amount of o-dianisidine to HRP-II, and spectrum #4 : 170s after.

conformational change of the HRP-II-o-dianisidine complex (3,28). The reaction scheme is given as (54):

$$\text{HRP-II} + \text{o-DH}_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (\text{HRP-II-o-DH}_2) \qquad (13)$$

$$(\text{HRP-II-o-DH}_2) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (\text{HRP-product}) \qquad (14)$$

Under pseudo-first order conditions and using the steady state assumption (54,55), it is shown in Appendix A that the rate constant for the formation of (HRP-product) is:

$$k(\text{obs}) = \frac{k_1[\text{o-DH}_2]\ (k_2 + k_{-2}) + k_{-2}k_{-1}}{k_1[\text{o-DH}_2] + k_{-1} + k_2} \qquad (15)$$

which can be rearranged into the following form:

$$k(\text{obs}) = \frac{k_1k_2[\text{o-DH}_2] + k_{-2}\{k_1[\text{o-DH}_2] + k_{-1}\}}{k_1[\text{o-DH}_2] + k_{-1} + k_2} \qquad (16)$$

If the substrate addition step in equation (13) is a rapid equilibrium, the rate equation becomes:

$$k(\text{obs}) = \frac{k_2K_1[\text{o-DH}_2]}{\{1^{\circ} + K_1[\text{o-DH}_2]\}} + k_{-2} \qquad (17)$$

where $K_1 = k_1/k_{-1}$: the equilibrium binding constant; k_2 and

k_{-2} are the forward and the reverse rate constants for the conformational or rearrangement of the enzyme-substrate complex, respectively. At low substrate concentrations, k(obs) will show a linear dependence upon [o-DH_2] :

$$k(obs) = k_2K_1[o\text{-}DH_2] + k_{-2} \qquad (18)$$

and at higher concentrations,

$$k(obs) = k_2 + k_{-2} \qquad (19)$$

Within rather large error limits, the rate constant k_{-2} can be assumed to be extremely small compared to k_2 since the extrapolation of the slope of the initial portion of the k(obs) versus [o-dianisidine] plots appears to go through the origin. Equation (17) becomes:

$$k(obs) = \frac{k_2K_1[o\text{-}DH_2]}{1 + K_1[o\text{-}DH_2]} \qquad (20)$$

For the purpose of computer weighted curve fitting of data, an estimated value of k_2K_1 was obtained from the slope of the initial portion of the curve. A value for K_1 was estimated by dividing the slope (k_2K_1) by the maximum value of k(obs) attainable at each experimental pH. Results from the computer fittings are presented in Table III.2. Values for the apparent second-order rate constant, k(app) (Table

III.3.), were calculated from the following equation:

$$k(app) = k_2K_1 \qquad (21)$$

The apparent second-order rate constant remained constant between pH 4.47 - pH 7.90 and decreased with increasing the pH above 8.0 (Figure III.11.).

Table III.2.

Values of K_1 and k_2 for the reaction of Compound II with o-Dianisidine at 10.0±0.2°C and I=0.11M

pH	BUFFER(c)	$K_1(M^{-1})$ (a)	$k_2(s^{-1})$(b)
4.47	CT	$(3.4\pm1.2)\times10^4$	$(2.2\pm0.3)\times10^3$
5.33	CT	$(5.1\pm2.8)\times10^4$	$(1.6\pm0.2)\times10^3$
6.49	P	$(2.3\pm1.4)\times10^5$	$(7.7\pm2.6)\times10^2$
7.20	P	$(2.8\pm0.8)\times10^5$	$(7.6\pm1.1)\times10^2$
7.60	P	$(4.7\pm0.7)\times10^5$	$(5.5\pm0.6)\times10^2$
8.20	P	$(1.3\pm0.6)\times10^6$	$(1.9\pm0.7)\times10^2$
9.00	C	$(5.8\pm0.6)\times10^5$	$(9.6\pm0.9)\times10$
9.56	C	$(1.2\pm0.6)\times10^6$	$(4.5\pm2.0)\times10$
9.80	C	$(3.6\pm0.7)\times10^5$	$(2.3\pm0.3)\times10$
10.60	C	$(7.2\pm1.0)\times10^5$	$(1.4\pm0.2)\times10$

(a) $K_1 = k_1 / k_{-1}$;

(b) k_2:conformational /rearrangement rate constant for the enzyme-substrate complex;

(c) Buffer key:

CT: citric acid-sodium citrate,

P : potassium dihydrogen phosphate - disodium hydrogen phosphate, and

C : sodium bicarbonate -sodium carbonate.

Table III.3.

Values of k(app) as a function of pH for the reaction of HRP-II with o-Dianisidine.

pH	k(app) ($M^{-1} s^{-1}$)	log k(app)
4.47	$(6.3\pm0.7)\times10^{7}$	7.80
5.33	$(6.4\pm0.8)\times10^{7}$	7.81
6.49	$(7.8\pm2.5)\times10^{7}$	7.89
7.20	$(9.8\pm3.6)\times10^{7}$	7.99
7.60	$(7.1\pm2.5)\times10^{7}$	7.85
8.20	$(3.7\pm1.3)\times10^{7}$	7.57
9.00	$(1.2\pm0.6)\times10^{7}$	7.07
9.56	$(9.4\pm2.7)\times10^{6}$	6.97
9.80	$(2.9\pm0.9)\times10^{6}$	6.46
10.60	$(2.4\pm0.8)\times10^{6}$	6.38

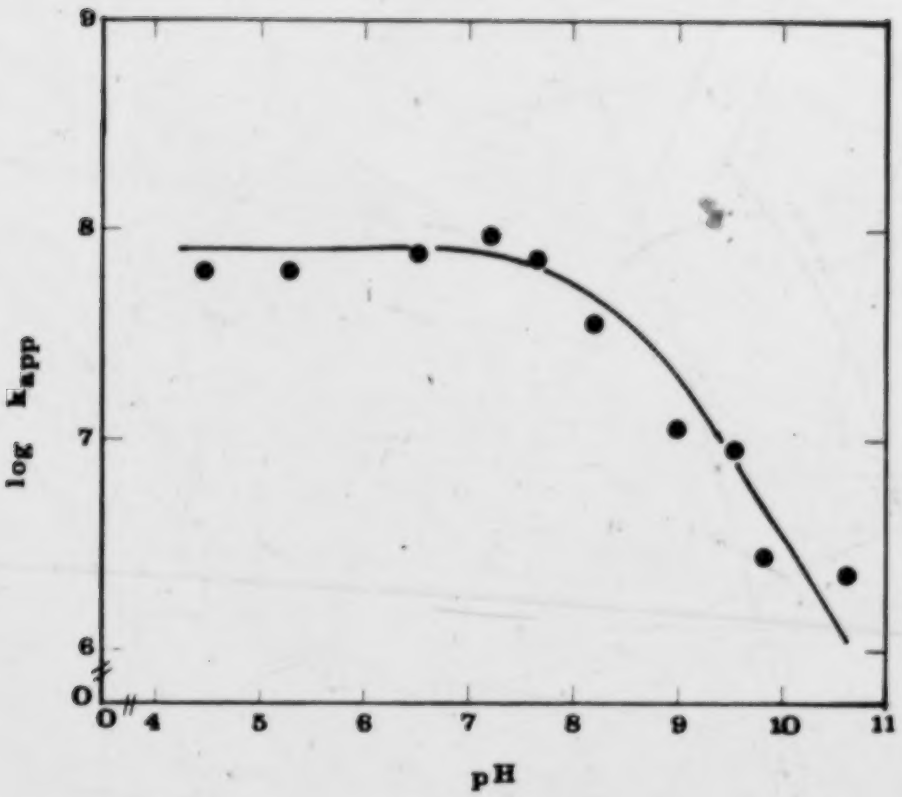

Figure III.11. Log k(app) versus pH profile for the reaction of o-dianisidine with HRP-II of horseradish peroxidase. The best-fit line was determined by weighted non-linear least squares analysis using equation 27 .

IV. DISCUSSION

The overall reaction for the peroxidation of o-dianisidine by horseradish peroxidase was given in the following scheme (46):

$$H_2\ddot{N}\ Ar\ \ddot{N}H_2 + (Fe(IV)\overset{+}{\ddot{\pi}}) \rightleftharpoons \{H_2\ddot{N}\ Ar\ \ddot{N}H_2, (Fe(IV)\overset{+}{\ddot{\pi}}\} \quad (22)$$

$$\{H_2\ddot{N}\ Ar\ \ddot{N}H_2, (Fe(IV)\overset{+}{\ddot{\pi}})\} \longrightarrow \{H_2\ddot{N}\ Ar\ \overset{+}{\dot{N}}H_2\ , (Fe(IV)\ddot{\pi}\} \quad (23)$$

$$\{H_2\ddot{N}\ Ar\ \overset{+}{\dot{N}}H_2, (Fe(IV)\ddot{\pi})\} \xrightarrow{\text{slow}} \{\ HN{=}Ar{=}NH, (Fe(III)\ddot{\pi}H_2{}^{2\cdot}\} \quad (24)$$

$$\{HN{=}Ar{=}NH, (Fe(III)\ddot{\pi}H_2{}^{2\cdot})\} \longrightarrow HN{=}Ar{=}NH + (Fe(III)\ddot{\pi}H_2{}^{2\cdot}) \quad (25)$$

where $H_2\ddot{N}\ Ar\ \ddot{N}H_2$ is the substrate o-dianisidine indicating the groups involved in the oxidation process; HN=Ar=NH is the first free product of the peroxidation of o-dianisidine which is dianisidine quinonediimine (the two-equivalent oxidized form (34)); and ($Fe(IV)\overset{+}{\ddot{\pi}}$) is the electronic structure representation of compound I of horseradish peroxidase taken to be the Fe(IV) π-cation radical (58).

The calculated rate constant for the reaction of HRP-I with o-dianisidine was in the order $10^8\ M^{-1}s^{-1}$ which is approximately 100 times faster than that for the reaction of HRP-II and o-dianisidine. This may explain the fact that the

study of the reaction of HRP-I and o-dianisidine was not feasible under pseudo-first order conditions. Also, it may account for the limitation of the accessible pH range of study (pH 7.91 - pH 10.60) under second-order conditions where the rate of the reaction was found to be relatively slow. A futher experimental study of the reaction below pH 7.91 has indicated that the increase in the rate constant value was so high that it was not feasible to measure in the stopped-flow. Therefore, it was not possible to obtain a second-order plot of k(obs) versus [o-dianisidine] so as to ascertain whether or not the reaction of HRP-I with o-dianisidine exhibits the same type of saturation effect as the reaction of HRP-II with o-dianisidine.

In Figures III.9. and III.10., a broad shoulder at 450 - 550 nm was observed. The width of this shoulder decreased gradually with time and eventually was limited to the region from 450 -500 nm. This was noted to occur for the reaction between o-dianisidine and native horseradish peroxidase and was attributed to the formation of the final product biazobiphenyl (structure Figure I.4.) (λ(max) 453 -475 nm) (2).

In a study done on horseradish peroxidase, it was suggested that the enzyme may exist in two conformations (59). The first conformation is believed to be more sterically restrictive and is to be favoured in the native enzyme. The second conformation favoured by compound II of horseradish peroxidase is believed to facilitate the binding

of aromatic molecules to the enzyme. Evidence for the binding of the substrate to the enzyme in this study can be obtained from Figures III.9. and III.10. The formation of a complex is indicated by an increase in absorbance at 403 nm for the reactions starting with HRP-I (Figure III.9.) and HRP-II (Figure III.10.). A further close examination of this increase in the absorbance peak at 403 nm indicates it to be slightly more for the compound II reaction than for the compound I reaction.

It was observed from Figure III.11. that the apparent second-order rate constant remained contant below pH 7.60 and, above pH 8.0, a decrease in k(app) was noticed. The simplest reaction scheme that may account for such observations is:

$$\begin{array}{ccc} EH + S & \xrightarrow{k_3} & \text{products} \\ [H^+] \Big\uparrow \Big\downarrow K_a(E) & & \\ E & & \end{array} \qquad (26)$$

where E and EH are the basic and acidic forms of the enzyme with the ionization constant $K_a(E)$; k_3 is the second order rate constant for the reaction involving the protonated form of the enzyme, and S is the substrate. Within the pH range of the study, the effect of the ionization of the enzyme functional group was considered and not those for the amino groups of the substrate. The pK_a for the o-dianisidine was

reported to have a value of 3.24 (2) which lies outside the pH range of study. This supported the assumption that an overall charge of zero was appropriate for the reaction of o-dianisidine with horseradish peroxidase (2).

From equation 26, equation 27 was derived for the apparent rate constant showing its dependence upon the experimental pH:

$$k(app) = \frac{k_3}{1 + K_a(E)/[H^+]} \qquad (27)$$

The best-fit parameter values and the standard deviations obtained from a non-linear least-squares computer program are presented in Table IV.1.

In studies of the reaction of compound II with some substrates, the effect of an acid group of $pK_a = 8.6$ was was observed (24-26,29,48,59). From the results of this study ,the calculated pK_a value of 8.81 ± 0.18 was assigned to the same enzyme functional group. The ionization of $pK_a = 8.6$ has been assigned to an amino acid residue at the distal side of the heme probably His 42 (1,19). It was suggested that this distal positioned histidine was hydrogen bonded with the sixth ligand of the heme (60). This distal histidine has a catalytic function in the compound II reactions (1,60). This distal group in its protonated form is believed to promote electron transfer to the histidyl residue occupying the fifth coordination position of the heme (61). In a more

recent Raman study of ferric horseradish peroxidase (20), it was proposed that the ionization of the distal histidine group plays an essential role in peroxidase catalysis because it results in a significant change in the conformation of the polypeptide chain. This change can be observed as a strain exerted upon the Fe-His(proximal) bond on the opposite side of the heme.

From both the spectroscopic and kinetic data, it can be concluded that o-dianisidine forms a complex with compound II. The reaction of o-dianisidine with HRP-II can be represented by the following scheme:

$$\text{HRP-II} + \text{o-DH}_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \{\text{HRP-II-o-DH}_2\} \qquad (13)$$

$$\{\text{HRP-II-o-DH}_2\} \xrightarrow{k_2} \{\text{HRP-product}\} \qquad (24)$$

This scheme involves a step where the substrate binds to the enzyme in a fast equilibruim. This complex undergoes an irreversible conformational change followed by the separation of the first free product from the native enzyme. From this study, it appears that the conformational change is the rate limiting step of the overall reaction. The calculated pK_a value of 8.8± 0.2 in this study was assigned to the distal histidine residue of catalytic importance.

In summary, an earlier study of the reaction of horseradish peroxidase with o-dianisidine was conducted under steady-state conditions in which only the rate of the

product formation was measured. In this study, elementary rate constants for the reaction of both compounds I and II with o-dianisidine were obtained as a function of pH.

Table IV.1.

Kinetic parameters for the log k(app) versus pH profile for the reaction of HRP-II with o-Dianisidine.

$k_3(M^{-1}s^{-1})$	$(7.4\pm1.6)\times10^{7}$
$K_a(E)$ (M)	$(1.5\pm0.6)\times10^{-9}$
$pK_a(E)$	8.8 ± 0.2

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APPENDIX A: Derivation of equation 15

Considering the following reaction scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP* \qquad (A1)$$

$$[E_o] = [E] + [ES] + [EP*] \qquad (A2)$$

Therefore: $[E] = [E_o] - [ES] - [EP*]$ (A3)

From the steady state approximation:

$$-\frac{d[ES]}{dt} = 0 = k_1[E][S] + k_{-2}[EP*] - k_{-1}[ES] - k_2[ES] \qquad (A4)$$

Substituting equation A3 into equation A4 :

$$[ES] = \frac{k_1[E_o][S] - k_1[EP*][S] + k_{-2}[EP*]}{k_1[S] + k_{-1} + k_2} \qquad (A5)$$

$$\frac{d[EP*]}{dt} = k_2[ES] - k_{-2}[EP*] \qquad (A6)$$

Therefore;

$$\frac{d[EP*]}{dt} = \frac{k_2(k_1[E_o] - k_1[EP*][S] + k_{-2}[EP*])}{k_1[S] + k_{-1} + k_2} + k_{-2}[EP*]$$

which could be rearranged to the following form;

$$\frac{d[EP*]}{dt} = \frac{k_2k_1[E_o][S] - [EP*]\{k_2k_1[S] + k_{-2}k_1[S] + k_{-2}k_{-1}\}}{k_1[S] + k_{-1} + k_2}$$

Integrating the above equation yields:

$$\frac{k_1[S] + k_{-1} + k_2}{k_2k_1[S] + k_{-2}k_1[S] + k_{-2}k_{-1}} \ln B/A = -t \qquad (A6)$$

where

$$A = k_2k_1[E_o][S]$$

and

$$B = k_2k_1[E_o][S] - [EP^*]\{k_2k_1[S] + k_{-2}k_1[S] + k_{-2}k_{-1}\}$$

rearranging equation A6:

$$\ln B/A = -\frac{(k_1[S])(k_2+k_{-2})) + k_{-1}k_{-2}}{k_1[S] + k_{-1} + k_2} t$$

which is

$$\ln B/A = -k(obs)\, t$$

where

$$k(obs) = \frac{k_1[S](k_2+k_{-2}) + k_{-1}k_{-2}}{k_1[S] + k_{-1} + k_2}$$

APPENDIX B: Derivation of equation 27

For the reaction scheme in equation 28 :

$$[E_o] = [E] + [EH]$$

$$k(app)\ [E_o] = k_3[EH]$$

Substituting for $[E_o]$ in the equation for k(app):

$$k(app) = \frac{k_3[EH]}{[E] + [EH]}$$

which could be rearranged into the following form:

$$k(app) = \frac{k_3}{[E]/[EH] + 1}$$

since, $K_a(E) = \frac{[E][H^+]}{[EH]}$

therefore, $K_a(E)/[H^+] = [E]/[EH]$

$$k(app) = \frac{k_3}{K_a(E)/[H^+] + 1}$$